Behavioral Stress-Induced Activation of FoxO3a in the Cerebral Cortex of Mice

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Background: The transcription factor FoxO3a is highly expressed in brain, but little is known about the response of FoxO3a to behavioral stress and its impact in the associated behavioral changes.

Methods: We tested the response of brain FoxO3a in the learned helplessness (LH) paradigm and tested signaling pathways that mediate the response of FoxO3a.

Results: A single session of inescapable shocks (IES) in mice reduced FoxO3a phosphorylation at the Akt-regulating serine/threonine residues and induced prolonged nuclear accumulation of FoxO3a in the cerebral cortex, both indicating activation of FoxO3a in brain. The response of FoxO3a is accompanied by a transient inactivation of Akt and a prolonged activation of glycogen synthase kinase-3beta (GSK3β). Noticeably, FoxO3a formed a protein complex with GSK3β in the cerebral cortex, and the interaction between the two proteins was stronger in IES-treated mice. Inhibition of glycogen synthase kinase-3 was able to abolish IES-induced LH behavior, disrupt IES-induced GSK3β-FoxO3a interaction, and reduce nuclear FoxO3a accumulation. In vitro approaches further revealed that the interaction between GSK3β and FoxO3a was strongest when both were active; FoxO3a was phosphorylated by recombinant GSK3β; and glycogen synthase kinase-3 inhibitors effectively reduced FoxO3a transcriptional activity. Importantly, IES-induced LH behavior was markedly diminished in FoxO3a-deficient mice that had minimal FoxO3a expression and reduced levels of FoxO3a-inducible genes.

Conclusions: FoxO3a is activated in response to IES by interacting with GSK3β, and inhibition of GSK3β or reducing FoxO3a expression promotes resistance to stress-induced behavioral disturbance by disrupting this signaling mechanism.

Key Words: Akt, FoxO3a, GSK3β, learned helplessness, signal transduction, stress

FoxO3a is a subtype of mammalian FoxO transcription factors (1,2) that are identified as orthologs of the *C. elegans* DAF-16 (3). FoxO3a is active when it is localized in the nucleus (4–6), a process tightly regulated by posttranslational modification. Activation of Akt by trophic signals phosphorylates FoxO3a at Thr32, Ser253, and Ser315 residues, which promotes sequestration of FoxO3a from the nucleus to the cytosol by the chaperone protein 14-3-3, therefore inactivating FoxO3a (7–11). Besides responding to trophic signals, environmental and physiological stresses, such as oxidative stress, ultraviolet irradiation, and food restriction, can activate FoxO3a, a response regulated by Akt-independent mechanisms, such as Jun N-terminal kinase, acetyltransferase cyclic adenosine monophosphate response element-binding protein and p300, and sirtuin deacetylases (12–18).

FoxO3a is highly expressed and widely distributed in adult brain (2,4,19,20). Destructive brain insults, such as ischemia and epileptic seizures, have been shown to increase the level of active FoxO3a that acts to eliminate damaged neurons by apoptosis (21–23). However, it is less known if brain FoxO3a is only active during the extreme apoptotic insults or if FoxO3a has other functions in response to abnormal brain activity, such as behavioral stress.

Behavioral stress often induces mood-related behavioral disturbance in vulnerable individuals, such as depression (24,25), as a result of disturbed neurotransmission, brain gene expression, and neuroplasticity (26,27). We and others previously reported that neurotrophins phosphorylate and inactivate FoxO3a in neuronal cells (28,29). Enhancing serotonin neurotransmission in animal brain also strongly phosphorylates and inactivates brain FoxO3a (30), a result in agreement with findings in *C. elegans* that activation of serotonin receptors lead to inhibition of DAF-16 transcriptional activity (31). Furthermore, both the monoamine reuptake inhibitor antidepressant imipramine and the mood stabilizer lithium suppress FoxO3a activity in mouse brain via different mechanisms of action (30,32). In accordance with these findings, mice with FoxO3a deficiency have higher resistance to stress-induced despair behavior in the forced swim and tail suspension tests (30).

We therefore hypothesize that brain FoxO3a may be overactive in response to behavioral stress. In this study, we investigated the response of mouse brain FoxO3a to inescapable footshocks (IES) in the learned helplessness (LH) paradigm and examined the underlying mechanisms mediating the response of FoxO3a and the behavioral impact of FoxO3a.

Methods and Materials

In addition to the brief descriptions of methods below, detailed Supplemental Methods and Materials can be found online in Supplement 1.

Animals

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the experimental protocol using mice. Adult (10- to 12-week-old) male mice were used for all experiments. Glycogen synthase kinase-3 (GSK3) inhibitor BIP-135 (33,34) or saline was infused into the right cerebral ventricle of mice via a cannula once daily. Behavioral stress was induced by repeated IES (35,36). Escape latency and failure were recorded as described (37), and social interaction was tested with a modified protocol (38).

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Cells

Human SH-SY5Y neuroblastoma cells and human embryo kidney (HEK)-293 cells were used for adenovirus infection and DNA plasmid transfection of FoxO3a and glycogen synthase kinase-3beta (GSK3β) DNA constructs.

Bioassays

For brain protein assays, mice were sacrificed on day 1, day 3, and day 8 after IES (Figure S1A in Supplement 1). Proteins from homogenate and nuclear/cytosolic extracts of mouse cerebral cortex were prepared as described (30,39). Proteins were immunoblotted with antibodies to phosphorylated or total FoxO3a, Akt, GSK3β, glycogen synthase kinase-3alpha (GSK3α), and Jun N-terminal kinase-1 (JNK-1). Immunoprecipitation was performed using anti-FoxO3a and anti-GSK3β antibodies (40).

Bioluminescence resonance energy transfer assay (41) was performed in HEK cells transiently co-transfected with RLuc-FoxO3a and yellow fluorescent protein (YFP)-GSK3β or their mutants as described (40).

FoxO3a phosphorylation by GSK3β was performed in vitro using immunoprecipitated hemagglutinin (HA)-tagged wild-type FoxO3a and recombinant human GSK3β in the presence of ^{32}P-adenosine triphosphate (ATP).

FoxO3a transcriptional activity was measured by luciferase assay in HEK cells co-transfected with FoxO3a and 6xDBE-firefly/renilla dual-luciferase plasmids (32).

Gene expressions of *FoxO3a, Bim, p27,* and *GADD45* in the cerebral cortex of mouse brain were measured by quantitative real-time polymerase chain reaction using the TaqMan Universal PCR system (Life Technologies, Carlsbad, California).

Statistical Analysis

All data are presented as mean ± SEM. Statistical analyses were conducted using SigmaStat 3.0 (Aspire Software International, Ashburn, Virginia). All data were checked for assumptions of normal distribution and homogeneity of variances in study samples, and any unexplained outlier value greater than ±2 standard deviations from the mean of the group was excluded. For a two-group comparison, statistical analysis was performed using unpaired Student *t* test. For comparison of more than three experimental groups, one-way or two-way analysis of variance was used to test for significant differences ($p \leq .05$) between groups. Any significant difference detected by analysis of variance was followed by post hoc or intergroup comparisons using Tukey's, Holm-Sidak, or Tamhane's test.

Results

To induce behavioral stress, adult (10- to 12-week-old) male wild-type C57BL/6 mice were subjected to a single session of IES that included 180 repeated inescapable footshocks delivered at unpredictable shock durations and intervals. Control mice were exposed to the footshock apparatus for the same length of time without receiving IES. When behaviors were tested on post-IES day 1, IES-treated mice exhibited significantly longer escape latency than control mice and higher numbers of escape failure (77.6 ± 7.1% of IES-treated mice and 5.4 ± 3.6% of control mice failed more than 15 of the 30 escape trials) (Figure S1B in Supplement 1). In addition, IES-treated mice exhibited poorer social interaction when compared with control mice (Figure S1C in Supplement 1). When the duration of LH behavior after a single session of IES was tested along a 15-day period of time, the elevated escape latency peaked on post-IES day 1, gradually reduced to approximately 50% of maximal but significantly higher than control level between day 3 and day 8, and remained at a residual longer escape latency even during day 11 and day 15 (Figure S1D in Supplement 1).

The response of FoxO3a to IES was measured in the cerebral cortex, which is a major stress-responding brain area (42–44). Although the levels of phospho-T32-FoxO3a and phospho-S253-FoxO3a (representing the Akt-regulated inactive state of FoxO3a [8]) were not different between IES-treated and control mice on post-IES day 1 (Figure 1A), both were significantly lower in IES-treated mice than control mice on post-IES day 3. On post-IES day 8, there was no significant difference in phospho-T32-FoxO3a between IES-treated and control mice, but phospho-S253-FoxO3a showed a trend of reduction ($p = .057$) in IES-treated mice.

Since active FoxO3a locates in the nucleus, the levels of nuclear and cytosolic FoxO3a in the cerebral cortex were measured. On post-IES day 1, IES did not cause significant change of nuclear or cytosolic FoxO3a, whereas on post-IES day 3, nuclear FoxO3a in IES-treated mice elevated to a significantly higher level than in control mice and this was accompanied by a small but significant reduction of cytosolic FoxO3a (Figure 1B). Surprisingly, the nuclear FoxO3a remained at a significantly higher level in IES-treated mice than control mice on post-IES day 8, suggesting that IES causes a prolonged activation of FoxO3a in the cerebral cortex.

Since Akt-dependent FoxO3a phosphorylation was decreased following IES treatment, we measured the levels of phospho-T308-Akt and phospho-S473-Akt, which represent active Akt, to determine if Akt activity was decreased by IES. No significant difference of phospho-T308-Akt or phospho-S473-Akt between IES-treated and control mice was found on post-IES day 1, but on post-IES day 3, the levels of both phospho-T308-Akt and phospho-S473-Akt reduced significantly in IES-treated mice without a change of total Akt (Figure 2). The reduction, however, was no longer significant on post-IES day 8. Therefore, IES caused a transient inactivation of Akt, which is in accordance with the time of reduction in Akt-dependent FoxO3a phosphorylation. However, the transient reduction of Akt activity does not concur with the prolonged robust nuclear accumulation of FoxO3a, suggesting that other regulatory mechanisms may be involved.

We previously reported (37) that the sensitivity of developing IES-induced LH behavior is markedly enhanced in GSK3 knockin mice (45), which express constitutively active GSK3, a protein kinase that is also negatively regulated by Akt (46). We therefore measured the inhibitory serine phosphorylation and the total level of GSK3β and GSK3α, the two isoforms of GSK3. On post-IES day 1, there was no significant change of phospho-S9-GSK3β in the cerebral cortex when IES-treated mice were compared with control mice (Figure 3A). However, the level of phospho-S9-GSK3β was significantly lower in IES-treated mice than in control mice on post-IES day 3, but the level of total GSK3β did not change. Different from Akt phosphorylation, phospho-S9-GSK3β remained significantly lower in IES-treated than in control mice on post-IES day 8 and there was a trend of increase in total GSK3β ($p = .064$), both suggesting a prolonged active state of GSK3β, which has a similar time course as the prolonged elevation of nuclear FoxO3a. In contrast, there was no significant change of phospho-Ser21-GSK3α or total GSK3α at the tested post-IES days (Figure 3B).

Jun N-terminal kinase-1 was reported to activate FoxO3a during oxidative and physical stresses (12–14), but no significant changes of phosphorylated or total JNK-1 were found in the cerebral cortex after IES (Table S1 in Supplement 1).

With the prominent effect of IES inducing a concomitant activation of GSK3β and FoxO3a, we next tested if there is an interaction between GSK3β and FoxO3a in mouse brain when they respond to IES. Interestingly, not only were GSK3β and FoxO3a from the cere-